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Antiviral Activity of Arildone on Deoxyribonucleic Acid and Ribonucleic Acid Viruses

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Arildone (3 μg/ml) reduced the replication of murine cytomegalovirus, Semliki Forest virus, vesicular stomatitis virus, and coxsackievirus A9 by 64, 68, 94, and 98%, respectively. When the plaque reduction method was used to evaluate the antiviral effect for the viruses, a concentration of 3 to 5 μg/ml yielded a 50% reduction in plaque numbers. The effect of arildone on virus replication was greatest when the drug was present from the time of inoculation. The effectiveness decreased as the time interval from the inoculation of the virus to the addition of the drug increased. The removal of the drug from infected cells by washing readily reversed the effect, and viral replication resumed at a significant level. Infectivity of these viruses was not inactivated by the drug. Tissue culture cells used for viral growth and assay grew well in arildone (3 μg/ml), with cell yields that were comparable to those for cultures in the absence of drug. At 3 μg/ml there were minimal effects of the drug on the uptake of ^{3}H-labeled amino acids and [^{3}H]-thymidine into cells. Furthermore, incorporation of these precursors was not affected. However, there was a reduction in uptake of [^{3}H]uridine into the acid-soluble pool and a concomitant reduction in incorporation into acid-insoluble counts.

Arildone, 4-[6-(2-chloro-4-methoxyphenoxy) hexyl]-3,5-heptane-dione, has been demonstrated to be effective in inhibiting the replication of some ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) viruses at concentrations which did not exhibit any toxic effects on host cellular processes (1, 2, 5, 6). Recently Kuhrt et al. (5), using the Curtis strain of herpes simplex virus type 2 (HSV-2), demonstrated that the drug interferes with early events that occur in HSV-2-infected BSC_1 cells before 6 h postinfection (PI). McSharry et al. (6), using poliovirus type 2 (polio type 2) in HeLa cells, demonstrated that the drug is not virucidal and does not interfere with viral adsorption or penetration. The drug specifically inhibited the uncoating of poliovirus, thus preventing the virus-induced shut-off of host cell protein synthesis. The drug therefore appears to have great potential for use as a broad-spectrum antiviral drug.

These findings prompted us to study the effect of arildone on several other DNA and RNA viruses in vitro and thereby establish its possible usefulness as an antiviral agent in vivo. In this paper, the effects of the drug on murine cytomegalovirus (MCMV), HSV-1 (strain KOS), HSV-2 (strain 333), Semliki Forest virus (SFV), vesicular stomatitis virus (VSV), and coxsackievirus A9 (Cox-A9) are presented.

MATERIALS AND METHODS

Cells and viruses. Primary mouse embryo fibroblasts (MEF) were prepared by trypsinization of 15- to 18-day-old mouse embryos of the CD-1 strain (3). The cells were seeded in 35-mm plastic petri dishes or in 32-ounce (ca. 0.97-liter) Brockway bottles. These cells were used for replication of MCMV and SFV (4). LLC-MK_2 cells (rhesus monkey kidney) were used for replication of Cox-A9 virus. Vero cells were used for replication of VSV, polio type 2, HSV-1, and HSV-2. The growth medium for these cells was minimal essential medium containing 10% fetal calf serum (FCS) which had been inactivated at 56°C for 30 min, 0.225% sodium bicarbonate, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

MCMV (strain Smith) was obtained from American Type Culture Collection and propagated for 52 passages in secondary MEF cells. SFV (A774, MG 4519), obtained from Robert E. Shope, Yale Arbovirus Research Unit of Yale University Medical School, New Haven, Conn., was passaged once in mice and once in secondary MEF cells before use. Polio type 2 and VSV (strain Indiana) were passaged in Vero cells. HSV-1 (strain KOS) was provided by R. B. Tenser, of the Milton Hershey Medical Center, Hershey, Pa.; HSV-2 (strain 333) was obtained from Fred Rapp, Milton Hershey Medical School. These two herpes strains were passaged and assayed in Vero cells. Cox-A9 which had been passaged in LLC-MK_2 cells was used.

Infection of cell monolayers. For infection and plaque titrations, appropriate cell monolayers prepared in 35- or 60-mm tissue culture plastic petri dishes were used. To infect the cells with virus, the medium was removed from the monolayers and virus was adsorbed to cells for 1 h at 37°C by adding 1 ml of fluid containing sufficient virus to give the desired multiplicity of infection (MOI). In those instances, indicated appropriate concentrations of arildone were included in the adsorption fluid. At the end of adsorp-

tion, unadsorbed virus was removed and the monolayers were washed three times with Leibowitz L-15 medium, using the appropriate concentration of arildone. Fresh L-15 medium containing 5% FCS and various concentrations of arildone was added, and the cultures were incubated at 37°C. The term "0 h PI" refers to the end of the 1-h virus adsorption period. For time-of-addition experiments, medium without arildone was added at 0 h PI, and the appropriate volume of the drug at a concentration of 100 μg/ml was added to obtain the desired concentration. Drug reversal experiments were done by removing the culture fluid containing medium with arildone and washing the monolayers three times with Leibowitz L-15 medium containing 5% FCS. Virus yield was determined by plaque assay of infected monolayers after freezing and thawing twice.

The specific details of infection and drug treatment for the various viruses are as follows.

Polio type 2. Monolayers of Vero cells prepared in 60-mm plastic petri dishes were infected with polio type 2 at an MOI of 0.1 plaque-forming unit (PFU)/cell. The drug, at various concentrations, was present during virus adsorption (60 min at 37°C) and was maintained in the medium of the infected cells. The infected cells were harvested 24 h PI and frozen and thawed twice; then the virus yield was determined by plaque titrations.

VSV, HSV-1, and HSV-2. Vero cells were infected with HSV-1 or HSV-2 at an MOI of 1 PFU/cell and maintained in various concentrations of arildone. The drug was present from the initiation of viral adsorption (60 min at 37°C) until 24 h PI. Infected cells were harvested at 24 h PI and were frozen and thawed twice before plaque assay.

MCMV and SFV. MEF cells infected with MCMV or SFV, each at an MOI of 1 PFU/cell, were maintained in various concentrations of arildone. The rest of the procedure was the same as above except that MCMV-infected cells were harvested at 72 h PI.

Cox-A9. LLC-MK_2 cells were infected at an MOI of 1 PFU/cell, maintained in various concentrations of arildone, and harvested at 24 h PI.

Plaque assay methods. MCMV was assayed by methods already described by Kim et al. (3). SFV was plaqued by the same method described for MCMV except that the plaques were counted on the 2nd or 3rd day. For plaque assay of poliovirus, HSV-1, and VSV, the same method described for MCMV was used except that monolayers of Vero cells were infected and plaques were counted on the 3rd day, whereas for Cox-A9 virus, LLC-MK_2 cells were used.

Effect of Arildone on cell growth. MEF, Vero, and LLC-MK_2 were seeded at approximately 10^5 cells per 60-mm plastic petri dishes with arildone at 0, 1, 3, 5, and 8 μg/ml. Each day thereafter for 3 days, the cells were dispersed with trypsin and counted with a hemacytometer.

Transport of precursor into cells and synthesis of macromolecules. MEF, Vero, and LLC-MK_2 cell monolayers were prepared in 60-mm plastic petri dishes and maintained at 37°C for 24 h in various concentrations of arildone in a CO_2 incubator. The cells were then pulsed for 90 min by adding 125 μCi of ^{3}H-labeled amino acids, [^{3}H]thymidine, or [^{3}H]-uridine per plate. At the end of the pulse, the medium was removed from the plates and the cell monolayers were rinsed three times with cold phosphate-buffered saline. Trichloroacetic acid-soluble activities were determined by extracting the cell monolayer twice with 2 ml of cold 5% trichloroacetic acid. Acid-insoluble activities were determined by solubilizing the trichloroacetic acid-extracted monolayers with 4 ml of 0.1 N NaOH. A portion (100 μl) of each sample was then mixed with S¢INT-A aqueous scintillation fluid (Packard Instrument Co., Inc., Downers Grove, Ill.) and assayed for counts per minute in a Packard liquid scintillation spectrometer, model 3380.

RESULTS

Effect of arildone on virus replication. Arildone in concentrations that are not toxic to host cells has been previously reported to inhibit the replication of HSV-1, HSV-2, and polio type 2. These viruses were also included in our experiments to serve as positive controls. The relative susceptibilities of the replication of the various viruses to arildone were compared and summarized in Table 1. Polio type 2 replication in Vero cells was very sensitive to arildone in that greater than 90% of the viral replication was inhibited by 1 μg/ml. In the presence of the drug, poliovirus-induced cytopathic effect was not detectable. HSV-1 (strain KOS) was susceptible to arildone. In the presence of 3 μg of arildone per ml, the replication of HSV-1 was inhibited by 94%. HSV-2 (strain 333) was also tested in Vero cells under similar conditions. The replication of HSV-2 was inhibited by 98%. Under the identical testing conditions, the replication of VSV in Vero cells, when tested at 24 h PI, was also susceptible in that 96% inhibition was seen in the presence of arildone at 3 μg/ml.

With arildone at 3 μg/ml, the replication of MCMV and SFV was inhibited by 64 and 68%, respectively. Maintenance of arildone from 24, 6, 1 and 0.5 h before the initiation of MCMV and SFV adsorption did not increase the effectiveness of the drug. LLC-MK_2 cells infected with Cox-A9 were harvested at 24 h PI, and the virus yield was determined. In 3 μg of the drug per ml, 98% reduction in the virus yield was observed.

It should be noted that the infectivity of HSV-1, HSV-2, MCMV, and Cox-A9 in mixtures of virus and up to 10 μg of arildone per ml was not reduced after 1 h at 37°C (data not shown). Thus, there is no direct effect of the drug on these viruses.

Effect of Arildone on Virus Plaque Formation. The minimal inhibitory concentration of arildone which reduced viral plaque titer by 50% was determined by adding various concentrations of the drug to overlay medium (Table 2). The same concentration of arildone was also present during virus adsorption. The effects of various concentrations of the drug on plaque formation of polio type 2, HSV-1, HSV-2, SFV, VSV, MCMV, and Cox-A9 are shown (Table 2).

TABLE 1. *Effect of arildone on virus replication*

Arildone concn (μg/ml)	Virus yield (PFU/ml)						
	MCMV[a]	SFV[b]	VSV[c]	Polio type 2[d]	HSV-1[e] (KOS)	HSV-2[f] (333)	Cox-A9[g]
0	1.8×10^6 (100)[h]	6.8×10^8 (100)	6.0×10^8 (100)	5.2×10^7 (100)	2.5×10^7 (100)	9.0×10^5 (100)	6.0×10^7 (100)
1	1.2×10^6 (66)	4.2×10^8 (62)	3.0×10^8 (50)	6.8×10^6 (13)	1.2×10^7 (48)	2.0×10^5 (22)	6.4×10^7 (107)
2	1.2×10^6 (66)	3.8×10^8 (56)	2.0×10^8 (33)	8.8×10^4 (0.17)	3.6×10^6 (14)	8.0×10^4 (8.9)	1.2×10^7 (20)
3	6.6×10^5 (36)	2.2×10^8 (32)	2.4×10^7 (4)	7.2×10^4 (0.14)	1.5×10^6 (6)	1.3×10^4 (1.4)	1.3×10^6 (2.2)

[a] MOI = 1.0 PFU; virus yield determined at 72 h PI.
[b] MOI = 1.0 PFU; virus yield determined at 24 h PI.
[c] MOI = 0.01 PFU; virus yield determined at 24 h PI.
[d] MOI = 0.1 PFU; virus yield determined at 24 h PI.
[e] MOI = 1.0 PFU; virus yield determined at 24 h PI.
[f] MOI = 0.1 PFU; virus yield determined at 36 h PI.
[g] MOI = 1.0 PFU; virus yield determined at 24 h PI.
[h] Percentage of virus yield obtained in culture with no arildone is given in parentheses.

TABLE 2. *Effect of arildone on virus plaque formation*

Arildone concn (μg/ml)	PFU[a]						
	HSV-1 (KOS)	HSV-2 (333)	Polio type 2	MCMV	SFV	VSV	Cox-A9
0	44 (100)[b]	23.5 (100)	136 (100)	46 (100)	70 (100)	51 (100)	24 (100)
1	32 (73)	22 (94)	0	42 (91)	55 (78)	45 (88)	23 (96)
2	24 (55)	15 (64)	0	Not done	40 (57)	45 (88)	19 (79)
3	22 (50)	12 (51)	0	30 (65)	30 (43)	48 (94)	10 (42)
4	16 (36)	8 (34)	0	14 (30)	25 (36)	44 (86)	8 (33)
5	1 (2)	6.5 (28)	0	8 (17)	6 (8.6)	22 (43)	7 (29)

[a] Average of number of plaques on four monolayers. Arildone was present at the time of inoculation and in the agarose overlay medium.
[b] Percentage of plaques obtained in monolayers with no arildone is given in parentheses.

There was complete elimination in plaque formation of polio type 2 in the presence of 1 μg/ml. For other viruses, 50% plaque reduction was observed in the presence of between 3 and 5 μg of arildone per ml. The plaques sizes of Cox-A9, VSV, MCMV, SFV, and, to some extent, HSV-1 and HSV-2 were smaller and less distinct at an arildone concentration of 3 μg/ml or higher.

Effect of time of addition or removal of arildone on Cox-A9 replication. Various concentrations (0, 3, 5, and 6 μg/ml) of arildone were added at different times from 1 h before infection to 6 PI, and the virus yield was determined at 24 h PI. Arildone most effectively reduced the virus yield when added at −1 or 0 h (Table 3). When the drug was added at 1.5 or 6 h PI, there was a diminished effect.

The effect of removal of the drug on virus yield was determined. At 0, 1, and 3 h PI, infected monolayers were washed three times with L-15 medium plus 15% FCS. Complete medium without arildone was added, and the cultures were incubated for 25 h before virus assay. In these cultures, virus replication occurred at a level similar to that observed in untreated, infected cells.

Effects of arildone on growth of MEF, Vero, and LLC-MK$_2$ cells. The effects of various concentrations of arildone on the growth of MEF, Vero, and LLC-MK$_2$ cells are shown in Table 4. There was no effect of arildone (at 1 and 3 μg/ml) on the growth and appearance of MEF cells. In the presence of 5 μg/ml, the morphology of the MEF cells appeared to be normal; however, cell growth was slower (Table 4). In an arildone concentration of 8 μg/ml, the cells showed signs of degeneration. There were long filamentous projections and rounding of cells, and the increase in cell number by 72 h was markedly reduced.

The growth of Vero cells and LLC-MK$_2$ in 1- and 3-μg/ml concentrations of arildone was comparable to that of these cells grown in the absence of the drug. Drug concentrations of 5 and 8 μg/ml definitely showed an inhibition of growth of both cell lines.

Since an arildone concentration of 8 μg/ml contained 0.08% of dimethyl sulfoxide, the effect on growth of MEF, Vero, and LLC-MK_2 cells of medium containing only that concentration of dimethyl sulfoxide was tested. There was no apparent effect on the growth of MEF, Vero, or LLC-MK_2 cells (data not shown).

TABLE 3. *Effect of time of addition or removal of arildone on Cox-A9 replication*

Arildone concn (μg/ml)	Time of addition (h PI)	Time of removal (h PI)	Yield[a] (PFU/ml)
0	None		2.6×10^7 (100)[b]
3	−1		2.1×10^6 (8)
5	−1		1.0×10^6 (4)
6	−1		2.7×10^5 (1)
3	0		4.7×10^6 (18)
5	0		2.1×10^6 (8)
6	0		1.0×10^6 (4)
3	1.5		1.9×10^7 (73)
5	1.5		1.8×10^7 (69)
6	1.5		2.0×10^7 (77)
3	6.0		1.5×10^7 (58)
5	6.0		2.1×10^7 (81)
6	6.0		2.1×10^7 (81)
0			2.5×10^7 (100)
3		0	2.4×10^7 (96)
5		0	2.1×10^7 (84)
3		1	2.0×10^7 (80)
5		1	2.8×10^7 (112)
3		3	1.6×10^7 (64)
5		3	1.9×10^7 (76)

[a] Virus yield determined by plaque assay 24 h after infection.
[b] Percentage of virus yield obtained in cultures with no arildone is given in parentheses.

Transport of precursors into cells and synthesis of macromolecules. The effects of arildone on the transport of precursors and on macromolecular synthesis in MEF, Vero, and LLC-MK_2 cells as determined by trichloroacetic acid-soluble and -insoluble activities are shown in Table 5. Arildone at concentrations up to 3 μg/ml had minimal effects on the transport of

TABLE 4. *Effect of arildone on growth of MEF, Vero, and LLC-MK_2 cells*

Arildone concn (μg/ml)	No. of cells ($\times 10^5$)/plate[a] at: 1[b]	2	3
MEF			
0	1.0[b] (100)[c]	2.5 (100)	3.5 (100)
1	0.9 (90)	3.5 (140)	3.6 (103)
3	1.0 (100)	1.8 (72)	3.7 (106)
5	0.8 (80)	1.4 (56)	1.9 (54)
8	1.0 (100)	1.6 (64)	1.5 (43)
Vero			
0	1.2 (100)	2.0 (100)	2.3 (100)
1	1.1 (92)	1.3 (65)	1.9 (83)
3	1.3 (108)	1.6 (80)	2.2 (96)
5	1.0 (83)	1.2 (60)	1.0 (43)
8	1.0 (83)	1.2 (60)	1.0 (43)
LC-MK_2			
0	1.3 (100)	1.5 (100)	3.7 (100)
1	1.1 (85)	1.6 (107)	3.9 (105)
3	0.7 (54)	1.3 (87)	3.4 (92)
5	1.2 (92)	1.1 (73)	1.9 (51)
8	1.1 (85)	1.1 (73)	1.8 (49)

[a] Average of two plates.
[b] Days after seeding.
[c] Percentage of control is given in parentheses.

TABLE 5. *Effect of arildone on macromolecular synthesis of MEF, LLC-MK_2, and Vero cells*

Arildone concn (μg/ml)	Incorporation of label[a] (cpm $\times 10^3$): ^{3}H-amino acids		[^{3}H]uridine		[^{3}H]thymidine	
	Acid soluble	Acid insoluble	Acid soluble	Acid insoluble	Acid soluble	Acid insoluble
MEF						
0	2.92 (100)[b]	5.95 (100)	10.95 (100)	2.92 (100)	2.81 (100)	10.45 (100)
1	2.99 (102)	6.10 (103)	11.04 (101)	3.14 (108)	3.23 (115)	9.90 (95)
3	2.86 (98)	5.01 (84)	8.31 (76)	2.07 (71)	3.40 (121)	8.84 (85)
5	2.42 (83)	3.83 (64)	5.20 (47)	1.44 (49)	3.30 (117)	6.15 (59)
8	2.41 (83)	2.32 (39)	2.37 (22)	0.82 (28)	3.49 (124)	4.13 (40)
LLC-MK_2						
0	9.34 (100)	7.04 (100)	8.12 (100)	3.00 (100)	1.12 (100)	0.42 (100)
1	10.91 (117)	6.58 (93)	6.49 (80)	2.61 (87)	1.28 (114)	0.57 (136)
3	10.37 (111)	5.74 (82)	5.37 (66)	2.16 (72)	1.29 (115)	0.58 (138)
5	10.33 (111)	4.58 (65)	3.20 (39)	1.55 (52)	1.12 (100)	0.39 (93)
8	13.30 (142)	2.19 (31)	1.87 (23)	0.71 (24)	1.06 (95)	0.19 (45)
Vero						
0	5.15 (100)	2.09 (100)	5.37 (100)	3.04 (100)	2.07[c] (100)	0.85 (100)
1	5.35 (104)	2.02 (97)	5.39 (100)	2.79 (92)	2.16 (104)	0.78 (92)
3	5.08 (99)	2.10 (100)	3.57 (66)	1.67 (55)	2.96 (143)	1.36 (160)
5	5.06 (98)	1.53 (73)	1.56 (29)	0.39 (13)	2.19 (106)	1.48 (174)
8	1.62 (31)	0.11 (5.3)	0.16 (3)	0.05 (1.6)	0.93 (45)	0.22 (26)

[a] Average of two determinations.
[b] Percentage of untreated control is given in parentheses.
[c] $\times 10^2$.

^{3}H-labeled amino acids into the three cell types. There was a slight effect on the incorporation of ^{3}H-labeled amino acids in MEF and LLC-MK_2 cells at a concentration of 3 μg/ml. Arildone at 3 μg/ml did show an appreciable effect on both the transport and incorporation of [^{3}H]uridine in all three cell lines. In MEF cells, arildone at a concentration of 3 μg/ml produced a slight increase in transport of [^{3}H]thymidine and a slight decrease in its incorporation. In Vero and LLC-MK_2 cells, the same concentrations of arildone showed an increase in both [^{3}H]thymidine uptake and incorporation.

DISCUSSION

At concentrations of up to 3 μg/ml, arildone had a minimal effect on the growth of MEF, Vero, and LLC-MK_2 cells. The fact that virus synthesis resumed after removal of the drug showed that there was no permanent damage to the cell's virus-synthesizing machinery. However, there was an effect upon the cells at this concentration, as shown by the reduction in acid-soluble and acid-insoluble counts of [^{3}H]-uridine in the three cell types tested.

At 3 μg/ml, arildone reduced the synthesis of all viruses tested. The effect was small, less than 1 $\log_{10}$ unit, for MCMV and SFV. The most pronounced reduction was seen with polio type 2, in which there was almost a 99% decrease in virus yield. VSV, HSV-1, HSV-2, and Cox-A9 were affected at an intermediate level, with a 90 to 99% yield decrease. Thus, arildone in in vitro systems appears to have antiviral activity at concentrations that have only minimal effect upon cellular morphology and cellular macromolecular synthesis.

Further studies are being done on various aspects of the antiviral activity of arildone and several related drugs.

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